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Role of proteoglycans and cytoskeleton in the effects of TGF-β1 on renal proximal tubule cells

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Role of proteoglycans and cytoskeleton in the effects of TGF-β1 on renal function. Transforming growth factor-beta (TGF-β) is a critical cell regulatory protein which influences cell growth, cell differentiation and cell chemotaxis. TGF-β1 has been previously shown to promote a migratory and adherent transformation of monolayers of renal proximal tubule cells in primary culture to form solid clusters of cells. To better understand the cellular basis of this TGF-β1 effect, these studies evaluated the influence of TGF-β1 on the synthesis of proteoglycans and on cytoskeleton rearrangement in rabbit renal proximal tubule cells in primary culture, and their role in this transformation effect of TGF-β1. Biosynthetic labeling of proteoglycans with ^{35}S sulfate and enzyme digestion studies demonstrated that TGF-β1 promoted the synthesis of heparan sulfate proteoglycans in these cells. The importance of proteoglycan synthesis induced by TGF-β1 in this migration and aggregation process was demonstrated with the use of two chemically-dissimilar proteoglycan synthesis inhibitors: xyloside and galactosamine. Both compounds inhibited TGF-β1 stimulation of proteoglycan synthesis and diminished TGF-β1 promoted transformation of proximal tubule cells as assessed by quantitative morphometry. Further experiments evaluated the influence of TGF-β1 on actin microfilaments with the use of rhodamine conjugated phalloidin staining and immunofluorescent microscopy, and demonstrated that TGF-β1 provoked a dramatic rearrangement of actin microfilaments into stress fibers. The use of actin microfilament disrupting agents, cytochalasin B and D, attenuated the stress fiber formation promoted by TGF-β1 and inhibited the TGF-β1-induced morphologic transformation of these cells. Further studies evaluated these effects on the rate of DNA synthesis in these cells, as assessed with ^{3}H-thymidine incorporation. Proteoglycan synthesis inhibitors significantly diminished the maximal proliferative response of these epithelial cells to epidermal growth factor (EGF). In contrast, actin microfilament disaggregation with cytochalasin B or D did not change the rate of DNA synthesis in response to EGF but did attenuate the antiproliferative effect of TGF-β1 on EGF-induced DNA synthesis cells. These studies demonstrate that the TGF-β1 promoted an increase in the production of proteoglycans and a higher ordered structure of the cytoskeleton. Both effects were instrumental in the adhesive migratory response of proximal tubule cells to TGF-β1 as well as the DNA synthesis rate response to both EGF and TGF-β1.

Transforming growth factor-beta (TGF-β) consists of a family of polypeptides with multiple functions in growth and differentiation [1–3]. To date, three mammalian isoforms have been identified: TGF-β1, β2, and β3. An impressive feature of this family of regulatory polypeptides is its multifunctionality. Depending on the cell type and cell environment, TGF-beta polypeptides can act as differentiating or proliferating factors [4–7]. Accordingly, this family of regulatory polypeptides has been demonstrated to be important in embryonic morphogenesis [8, 9], wound healing [10] and inflammatory processes [11, 12].

Recent observations have demonstrated that TGF-β1 has dramatic effects on renal proximal tubule cells in primary cell culture to alter growth responses under both basal and growth stimulating conditions [13–15]. In addition, TGF-β1 promoted a dramatic phenotypic alteration of the epithelial monolayer to transform into solid clusters of adherent cells [14], a process highly reminiscent to the initial inductive step of tubulogenesis in embryonic kidney morphogenesis [16].

To further understand the cellular basis of the TGF-β1 promoted effects on renal proximal tubule cells, the present series of experiments were undertaken. Since TGF-β has a dramatic effect on extracellular matrix production [17, 18], the effect of TGF-β1 on proteoglycan and fibronectin synthesis by renal proximal tubule cells and the functional role of these matrix components on the observed responses to TGF-β1 were evaluated. Since one of the effects of TGF-β1 is also characterized not only by increased cell adhesion but also by cell migration, the effect of TGF-β1 on cytoskeletal rearrangement in renal tubule cells and the functional role of microfilament aggregation on this TGF-β1 promoted processes were also assessed. The results of these experiments do, indeed, suggest an important role for proteoglycan synthesis and cytoskeletal rearrangement on the effects of TGF-β1 on renal tubule cells *in vitro*.

Methods

Cell culture

Rabbit renal proximal tubule cells were grown in primary culture by previously reported techniques [14]. The cells were grown in 35 mm. Corning culture dishes with serum free, hormonally defined DME, Hams F-12 media (1:1, vol/vol) containing glutamine, penicillin, streptomycin, 50 nM hydrocortisone, 5 μg/ml insulin, and 5 μg/ml transferrin. The cultures were maintained in a humidified 5% CO_2/95% air incubator at

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37°C. Media were changed every three to five days. Cultures became confluent in 9 to 12 days.

Biosynthetic labeling

Cultures were grown to confluence and were made quiescent by the addition of fresh medium containing no hydrocortisone and insulin and with low sulfate content for ^{35}S-sulfate labeling studies or with low methionine content for ^{35}S-methionine labeling. Cells were pretreated with 4-methylumbelliferyl-β-D-xyloside (xyloside, 1 mM), galactosamine (2.5 mM) or sham solution for 6 to 24 hours at which time TGF-β1 (10 ng/ml) was added. Cells were then exposed to TGF-β1 for 48 hours. This dose and exposure time were chosen after preliminary data demonstrated that these were maximal and optimal in assessing the measured parameters. Furthermore, under these conditions, TGF-β1 did not change cell numbers in the confluent plates. Eighteen hours prior to the termination of the experiment, ^{35}S-sulfate (200 μCi/ml) or ^{35}S-methionine (150 μCi/ml) was added to the cultures. Culture media and cell layers were harvested and processed for SDS-PAGE with fluorography as previously described [18]. Proteoglycans were identified by enzyme digestion and matrix proteins by immunoprecipitation as previously reported [18, 19]. Enzymatic digestion to identify proteoglycans was performed on conditioned media after biosynthetic labeling. Aliquots of medium (25 μl) were mixed with 100 milliunits of chondroitinase ABC or chondroitinase AC both in 100 mM Tris-HCl, pH 7.5, 10 mM calcium acetate, 2 mg/ml BSA or 100 milliunits of heparinase III in 50 mM Tris-HCl, pH 7.4, 1 mM calcium chloride, 5 mM calcium acetate. All samples also received 1 mM PMSF, 5 mM benzamidine, 100 μg/ml soy bean trypsin inhibitor, 10 μg/ml leupeptin and 10 μg/ml antipain. Chondroitinase-containing mixtures were incubated at 37°C for 1.5 hours, and heparinase containing mixture at 22°C for 1.5 hours. At termination samples were prepared for SDS-PAGE.

Immunofluorescence microscopy

After various treatment conditions, proximal tubule cells were prepared for immunofluorescence studies. Cells were fixed with 0.25% glutaraldehyde in phosphate buffered saline (PBS), pH 7.3, for one hour at room temperature or overnight in a refrigerator, then permeabilized with 0.1% Triton X-100 in PBS for five minutes. Actin microfilaments were visualized by staining with phalloidin conjugated to rhodamine diluted 1:30 (950 μl/plate) for 30 minutes at 37°C. Ten μl of 90% glycerol was then added. Preparations were examined with a Zeiss photomicroscope equipped for transmitted light and epifluorescence.

Quantitative morphometry

This process was accomplished by previously described techniques [14]. In brief, two random fields were selected from each culture plate. At 100× magnification, 8 × 10 inch photographic print was made of the selected field. Using a computer operator assisted morphometer (Woods Hole Educational Associates, Woods Hole, Massachusetts, USA) and a Summa graphics digitizing tablet, the area of field occupied by cells (A) and that not occupied with cells (B) were measured. The percent of the culture plate which was transformed after TGF-β1 addition, (T), was calculated at $T(\%) = B/(A + B)$.

^{3}H-thymidine incorporation

The DNA synthetic response of cells to various agents was monitored using ^{3}H-thymidine incorporation. Confluent cultures were made quiescent by the addition of fresh medium containing no hydrocortisone or insulin. After 24 hours, xyloside (1 mM), galactosamine (2.5 mM) or cytocholasin B (0.4 μM) or D (0.02 μM), was added to the culture media. After an additional six hours, TGF-β1 (10 ng/ml) or sham solution was added. After 48 hours of TGF-β1 exposure, EGF (0.1 μM) was added. ^{3}H-thymidine was added to the cultures and the cells were processed for ^{3}H-thymidine incorporation 24 hours later by methods previously reported [14].

Statistical methods

Statistical analysis was performed using Student's *t*-test. Unless otherwise stated, the number of experiments refer to a separate number of cultures. All results are represented as mean ± SE.

Materials

All reagents used were of the highest grade commercially available. All organic reagents were obtained from Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise indicated. Radioactive tracers were obtained from New England Nuclear (Boston, Massachusetts, USA): (Methyl-^{3}H)-thymidine (2 Ci/mmol), ^{35}S-methionine (1139 Ci/mmol), ^{35}S-sulfate (748 μCi/mmol). EGF (recombinant human) was obtained from Amgen Biologicals (Thousand Oaks, California, USA), TGF-β1 (porcine platelets) from R & D Systems (Minneapolis, Minnesota, USA). Rhodamine-conjugated phalloidin was purchased from Molecular Probes (Junction City, Oregon, USA). Polyclonal antibodies against fibronectin were obtained as previously described [18].

Results

Characterization of TGF-β1-induced proteoglycan synthesis

Biosynthetic labeling with ^{35}S-sulfate of proteoglycans produced by primary rabbit renal proximal tubule cells under control, quiescent, confluent conditions demonstrated that at least two classes of proteoglycans were synthesized and secreted into the culture media (Fig. 1). One class was identified as a broad band on SDS-PAGE centered at 220 kD and another as a labeled band at the top of the lane due, most likely, to restrictive entry into the gel from the large size of this molecule. Treatment of cells with TGF-β1 for 48 hours increased the quantity of secreted proteoglycans, as reflected by the increased density of the proteoglycan bands on SDS-PAGE. In three independent experiments, this increase in proteoglycan production was approximately threefold. Exposure of quiescent, confluent cells in culture to the proteoglycan synthesis inhibitor [20], 4-methylumbelliteryl-β-D-xyloside (xyloside, 1 mM) 6 to 24 hours prior to TGF-β1 treatment diminished the labeling of both classes of secreted proteoglycans both under control and TGF-β1 stimulated conditions. Increased labeling of small molecular weight molecules (less than 66 kD) was also observed. These components are most likely glycosaminoglycans (GAG) due to the primary action of xyloside to compete with the xylosylated core protein for GAG chain elongation,

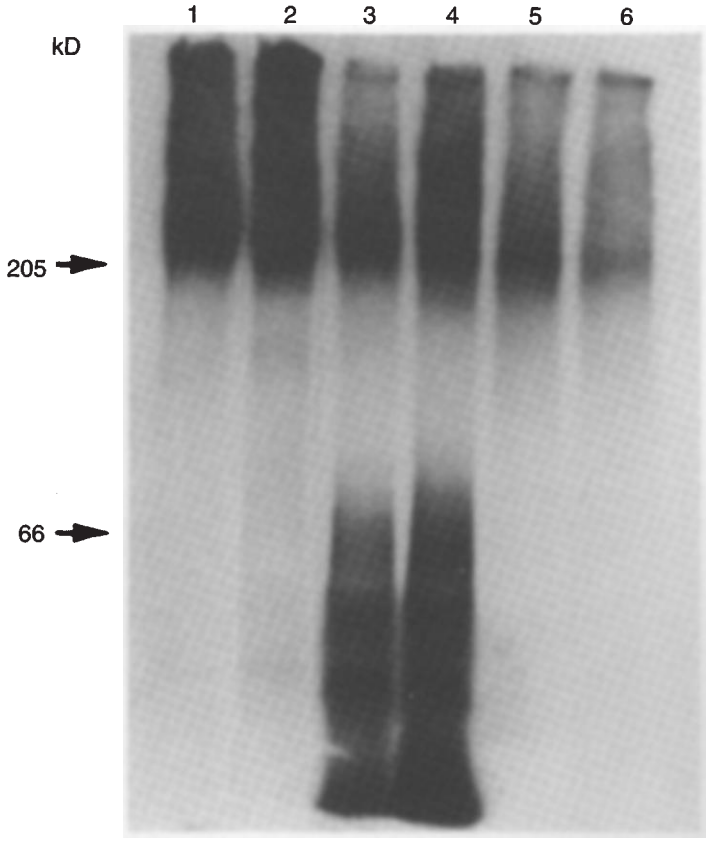

Fig. 1. *Effect of TGF-β1 on synthesis of proteoglycans secreted into culture media by proximal tubule cells as demonstrated in this representative experiment.* Cell cultures were treated with various agents and metabolically labeled with ^{35}S-sulfate. Equal volumes of culture media were analyzed by SDS-PAGE and fluorography. Lane 1, control; Lane 2, TGF-β1; Lane 3, xyloside; Lane 4, xyloside and TGF-β1; Lane 5, galactosamine; Lane 6, galactosamine and TGF-β1.

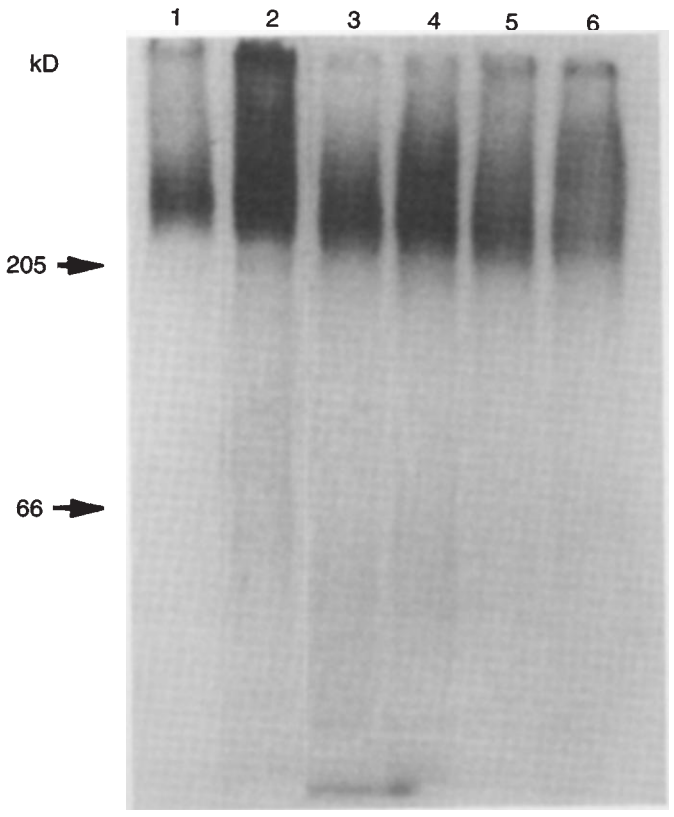

Fig. 2. *Effect of TGF-β1 on synthesis of proteoglycans incorporated into the cell layer as demonstrated in this representative experiment.* Cell cultures were treated with various agents and metabolically labeled with ^{35}S-sulfate. Equal amounts of cellular extracts were analyzed by SDS-PAGE and fluorography. Lane 1, control; Lane 2, TGF-β1; Lane 3, xyloside; Lane 4, xyloside and TGF-β1; Lane 5, galactosamine; Lane 6, galactosamine and TGF-β1.

thereby uncoupling proteoglycan synthesis [21]. Similarly, pretreatment of cells with galactosamine (2.5 mM), which acts as a sink for uridine diphosphate (UDP) which is a necessary factor for saccharide moieties to be utilized for proteoglycan synthesis [22], also diminished proteoglycan synthesis under both control and TGF-β1 treated conditions.

Assessment of ^{35}S-sulfate labeling of proteoglycans incorporated into the extracellular matrix by extraction and parallel analysis of the cell layer demonstrated that TGF-β1 increased the accumulation within the cell layer of radiolabeled proteoglycans of similar size as those secreted into the culture media. Xyloside and galactosamine were effective in diminishing the TGF-β1 related increase in newly synthesized proteoglycans incorporated into the cell layer (Fig. 2). Of note, the increases in GAG labeling released into the cell supernatant was not observed in the cell layer.

Identification of proteoglycans by enzyme digestion

Digestion of the conditioned media after biosynthetic labeling with ^{35}S-sulfate with glycosaminoglycan-degrading enzymes was accomplished to identify the type of proteoglycans produced under control and TGF-β1 stimulated conditions [18, 19]. Treatment of cell supernatants with heparinase III degraded virtually all ^{35}S-sulfate proteoglycans from both control and TGF-β1 treated conditions (Fig. 3); the same effects were observed with nitrous acid treatment. In contrast, treatment with chondroitinase AC and chondroitinase ABC did not have dramatic degradative effects on labeled proteoglycans under both control and TGF-β1 stimulated conditions. These results indicate that the proteoglycans produced by proximal tubule cells and augmented by TGF-β1 are heparan sulfate proteoglycans.

Effect of TGF-β1 on fibronectin synthesis

Biosynthetic labeling with ^{35}S-methionine of proximal tubule cells was employed to label newly synthesized proteins. Compared to control preparations, treatment with TGF-β1 (10 ng/ml) for 48 hours resulted in increases in the radiolabeling of several protein bands both in the cell medium and cell layer (data not shown). Since TGF-β1 has been shown to increase the synthesis of various extracellular matrix proteins, predominantly fibronectin [23], further experiments were done to determine whether TGF-β1 also stimulated fibronectin synthesis in renal proximal tubule cells. Parallel immunoprecipitation experiments with anti-fibronectin antibody followed by SDS-PAGE were carried out on radiolabeled condition media from both

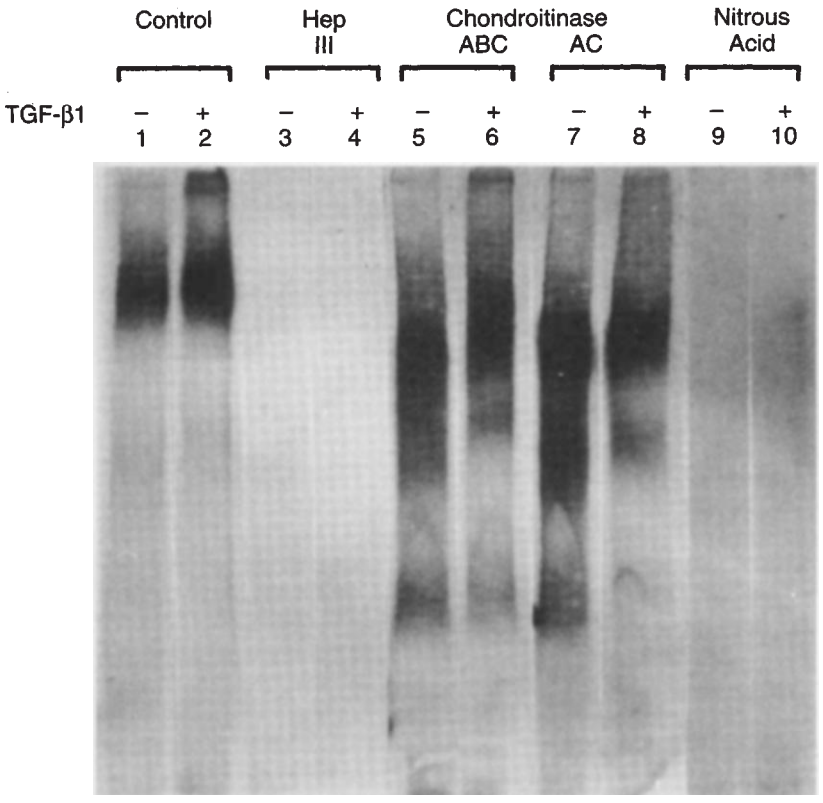

Fig. 3. *Identification of proteoglycans regulated by TGF-β1 in proximal tubule cells as demonstrated in this representative experiment.* Conditioned media containing ^{35}S-sulfate labeled proteoglycans were subjected to specific enzyme digestion. Conditioned media were obtained from cells treated (+) or not treated (−) with TGF-β1 for 48 hours. Conditioned media were treated with various enzymes as specified (Hep III represents heparinase III) then analyzed by SDS-PAGE and fluorography.

control and TGF-β1 stimulated cells. As demonstrated in Figure 4, renal proximal tubule cells synthesized fibronectin under control conditions; TGF-β1 treatment resulted in stimulation of fibronectin production. Similar results were observed in the extracted cell layer as well (data not shown). Of note, control preparations using pre-immune serum did not demonstrate precipitable product.

Effect of TGF-β1 on actin filament organization

As revealed by rhodamine-conjugated phalloidin staining in Figure 5, proximal tubule cells under control culture conditions contained both peripheral bands of actin near cell membranes and randomly oriented cytoplasmic fibers, similar to other epithelial cells [24, 25]. Treatment of cells with TGF-β1 resulted in higher ordered actin filament architecture which progressed with time. As early as 8 to 12 hours after TGF-β1 (10 ng/ml) addition to quiescent confluent monolayers of proximal tubule cells, actin fibers coalesced into large bundles, or stress fibers, with direct extension of these fiber formations from cell to cell, indicating that these bundles share spatial information. By 24 hours, relatively intense staining of actin was observed at sites of cellular aggregates where actin fibers were forming a tight lattice network among cells. At 48 hours, the actin filaments have organized into a three dimensional web supporting the structure of the aggregate; and at 72 hours, clearly defined adhesion plaques and stress fibers in cell surface protrusions were evident. Both cytochalasin B (0.4 μM) and cytochalasin D (0.02 μM) treatment of cells resulted in disruption of microfilaments with localized aggregation (Figs. 5 C and D). Under control conditions, cytochalasin treatment rapidly promoted dense aggregation of actin filaments at the junction of cells, the sites of fiber initiation and communication between cells. The rearrangement of actin filaments into stress fibers and orchestrated architectural arrays between cells induced by TGF-β1 was completely inhibited by cytochalasin B and D at 12 hours of exposure.

Role of proteoglycans and actin in TGF-β1 promoted phenotypic transformation

Previous reported experiments have demonstrated that TGF-β1 induces a phenotypic transformation of monolayers of renal proximal tubule cells into migratory, adhesive aggregates of cells [14]. As demonstrated in Figure 6, this transformation, as measured by quantitative morphometry, began approximately 12 hours following TGF-β1 exposure with completion at 72 hours. Since the current data demonstrate that TGF-β1 promotes synthesis of various extracellular matrix molecules, including proteoglycans and fibronectin, and actin microfilament rearrangement in renal proximal tubule cells, further experiments utilizing quantitative morphometry were undertaken to assess the effects of proteoglycan synthesis inhibition and microfilament disruption on this TGF-β1 promoted transformation. As seen in Figure 6, pretreatment of the cell monolayer with xyloside (1 mM) and galactosamine (2.5 mM), as proteoglycan inhibitors, for 6 to 24 hours prior to TGF-β1 (10 ng/ml) resulted in a delayed and inhibitory transformation response to TGF-β1. Since galactosamine inhibits proteoglycan metabolism by acting as a UDP sink to limit the availability of UDP as a necessary factor for GAG extension, uridine has been previously shown to reverse the metabolic effects of galactosamine [7]. In an additional set of experiments from those

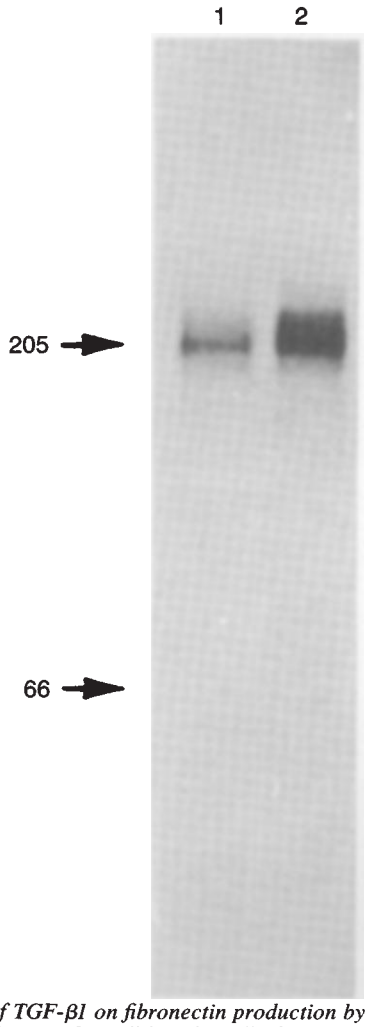

Fig. 4. *Effect of TGF-β1 on fibronectin production by proximal tubule cells.* Equal volumes of conditioned media from control (lane 1) and TGF-β1 (lane 2) treated cells were immunoprecipitated with antisera to fibronectin followed by SDS-PAGE and fluorography.

reported in Figure 6, uridine (5 or 15 mM) was added simultaneously with galactosamine to proximal tubule cells six hours prior to TGF-β1 treatment to determine if this compound could reverse the effect of galactosamine to inhibit TGF-β1 promoted transformation. In this regard, uridine reversed the inhibitory effect of galactosamine on TGF-β1 alterations. At 72 hours, by quantitative morphometry, TGF-β1 alone induced a 78 ± 6% transformation response; galactosamine pretreatment reduced this TGF-β1 effect to 21 ± 1% ($N = 3$, $P < 0.01$ compared to TGF-β1 alone); simultaneous uridine and galactosamine treatment resulted in a TGF-β1 transformation response of 44 ± 5% ($N = 3$, $P < 0.02$ compared to galactosamine + TGF-β1).

Pretreatment of cells with either cytochalasin B (0.4 μM) or cytochalasin D (0.02 μM) retarded TGF-β1 induced phenotypic transformation with a magnitude effect greater than that observed with proteoglycan inhibitors, as shown in Figure 6. Of importance, no effect was observed with the fibronectin binding site inhibitor [26], arg-gly-arp-ser (1 mg/ml, data not shown).

Influence of proteoglycan synthesis inhibitors and actin microfilaments on DNA synthesis

Recent work has demonstrated a direct correlation between TGF-β1 induced phenotypic alteration and its antiproliferative effect in proximal tubule cells [14], thereby suggesting that the cellular elements responsible for this morphologic change may also play a role in this antiproliferative effect. Since the present data demonstrated a role for both proteoglycan synthesis and actin rearrangement in TGF-β1's effect, further experiments were undertaken to evaluate the influence of these factors on DNA synthesis rates in proximal tubule cells. Pretreatment of quiescent, confluent monolayers of cells with either xyloside (1 mM) or galactosamine (2.5 mM) for either 6 to 24 hours had little effect on basal DNA synthesis rates, as assessed by ^{3}H-thymidine incorporation, but significantly inhibited the increase in DNA synthesis rates promoted by EGF (0.1 μM), as depicted in Figure 7. This inhibition did not appear to be due to increases in the GAG, heparan sulfate, which may occur under these conditions, since the addition of heparan sulfate (50 mM) did not change the magnitude of EGF-induced ^{3}H-thymidine incorporation in renal proximal tubule cells (data not shown), as has been described in renal mesangial cells [27]. In contrast, pretreatment of monolayers with cytochalasin B (0.4 μM) had no effect on either control or EGF-stimulated DNA synthetic rates (Fig. 7). Similar results were seen with cytochalasin D (0.02 μM).

Since cytochalasin B retarded TGF-β1-induced phenotypic transformation but did not inhibit EGF-promoted ^{3}H-thymidine incorporation, cytochalasin B could be used to further test the relationship between TGF-β1 mediated transformation and its antiproliferative effect. As demonstrated in Figure 8, TGF-β1 addition to quiescent, confluent cells for 24 hours prior to EGF treatment resulted in a significant inhibition of the increases in ^{3}H-thymidine incorporation promoted by EGF. Pretreatment of cells with cytochalasin B (0.4 μM) six hours prior to TGF-β1 addition, which attenuated the rate of TGF-β1 induced transformation, however, significantly ameliorated the inhibitory effect of TGF-β1 on EGF induced increases in ^{3}H-thymidine incorporation. Similar findings were also observed with cytochalasin D (0.02 μM).

Discussion

Transforming growth factor-beta (TGF-β) is now considered a critical cell regulatory protein with a wide spectrum of cellular effects. This family of peptides has diverse influences on cell growth, cell differentiation, and cell chemotaxis [1–3]. Accordingly, TGF-β has been shown to be important in wound healing, tissue remodeling and embryonic morphogenesis [8–12]. TGF-β has been generally viewed as a growth stimulatory factor for mesenchymal cells and a growth inhibitory factor for epithelial cells. Unlike most epithelia, renal tubule cells are mesodermally derived, so that the effects of TGF-β on this cell may be especially interesting. In this regard, recent work has shown that TGF-β treatment of renal proximal tubule cells in primary cultures inhibited both baseline and EGF-stimulated DNA synthesis after 48 hours of exposure but enhanced EGF-stimulated DNA synthesis at 24 hours [14]. Morphologic evaluation demonstrated that TGF-β1 promoted a dramatic phenotypic transformation of the epithelial monolayer with migration

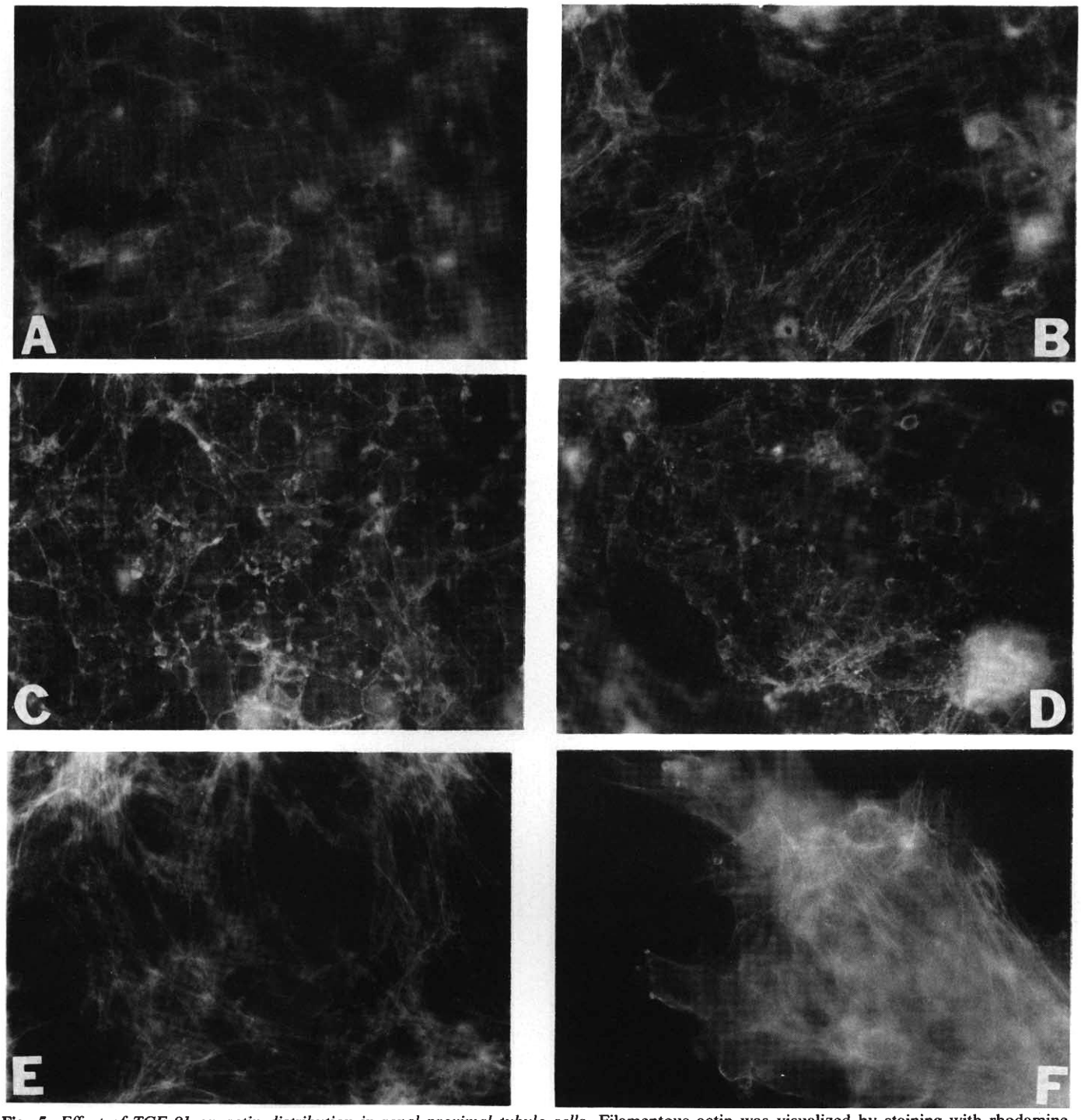

Fig. 5. *Effect of TGF-β1 on actin distribution in renal proximal tubule cells.* Filamentous actin was visualized by staining with rhodamine-phalloidin and indirect immunofluorescent microscopy. **A.** Monolayer of proximal tubule cells under control conditions. **B.** TGF-β1 treatment for 12 hours. **C.** Cytochalasin D exposure for 2 hours. **D.** Simultaneous TGF-β1 and cytochalasin D exposure for 12 hours. **E.** TGF-β1 treatment for 24 hours. **F.** TGF-β1 treatment for 72 hours.

and adhesion of the cells to form solid clusters of adherent cells. Quantitative morphometry demonstrated that this transformation developed within 12 hours after TGF-β1 exposure, was nearing completion after 48 to 72 hours of TGF-β treatment, and correlated to TGF-β1 related inhibition of EGF-induced DNA synthesis. Since many of the multiple actions of TGF-β1 are mediated through regulation of synthesis of extracellular matrix (ECM) components [17–23], these studies were undertaken to evaluate the influence of TGF-β1 on the synthesis of both proteoglycans and fibronectin, two key ECM molecules,

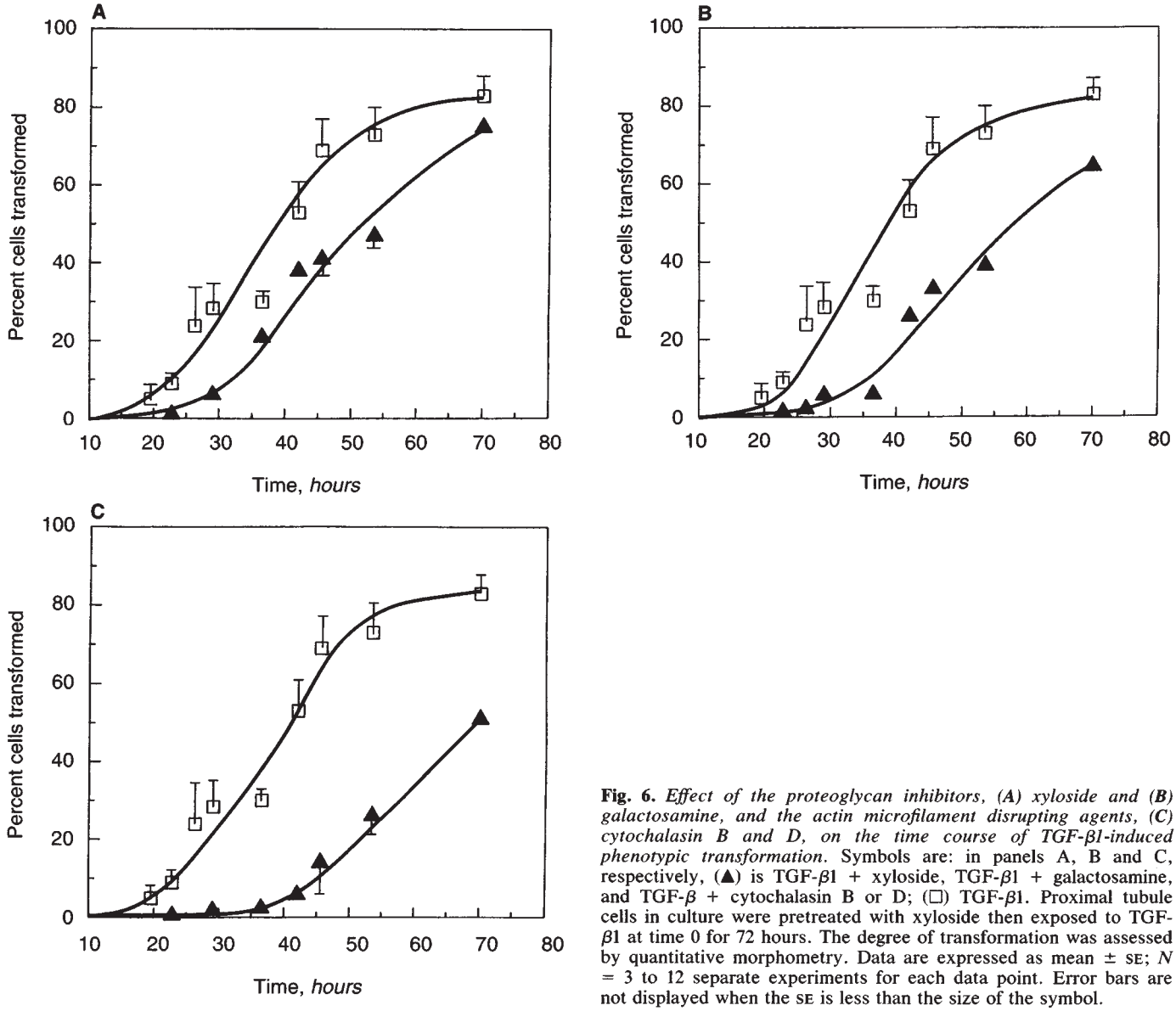

Fig. 6. *Effect of the proteoglycan inhibitors, (A) xyloside and (B) galactosamine, and the actin microfilament disrupting agents, (C) cytochalasin B and D, on the time course of TGF-β1-induced phenotypic transformation.* Symbols are: in panels A, B and C, respectively, (▲) is TGF-β1 + xyloside, TGF-β1 + galactosamine, and TGF-β + cytochalasin B or D; (□) TGF-β1. Proximal tubule cells in culture were pretreated with xyloside then exposed to TGF-β1 at time 0 for 72 hours. The degree of transformation was assessed by quantitative morphometry. Data are expressed as mean ± SE; *N* = 3 to 12 separate experiments for each data point. Error bars are not displayed when the SE is less than the size of the symbol.

and the role of these components in the functional effects of TGF-β1 action on renal proximal tubule cells.

The present findings demonstrate that TGF-β1 stimulated the synthesis of both proteoglycans and fibronectin by renal proximal tubule cells. Similar to a variety of other cells [23], including glomerular epithelial cells [19], proximal tubule cells synthesize fibronectin under basal conditions and this synthetic rate is increased with TGF-β1 exposure. Unlike other cells, including glomerular mesangial and glomerular epithelial cells, in which TGF-β1 promotes the synthesis of chondroitin/dermatan sulfate proteoglycans [11–14, 16–19], TGF-β1 stimulated the production of two classes of heparan sulfate proteoglycans. The synthesis of these proteoglycans appears to be important in the phenotypic transformation of proximal tubule cells which is promoted by TGF-β1. Two chemically dissimilar proteoglycan synthesis inhibitors were utilized in this study to test the functional role of proteoglycan synthesis on the effects of

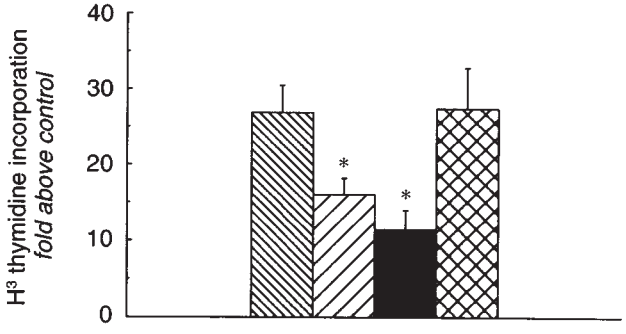

Fig. 7. *Effect of proteoglycan synthesis inhibitors and actin microfilament disruption on EGF-induced ^{3}H-thymidine incorporation (▨).* Pretreatment of tubule cells in culture with both xyloside (□) and galactosamine (■) inhibited EGF promoted DNA synthesis; cytochalasin B pretreatment (▩) had no effect. *$P < 0.02$ or better; $N = 6$ for each condition.

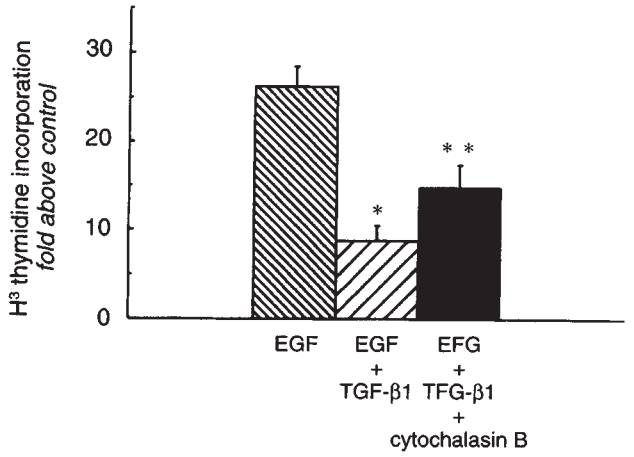

Fig. 8. *Effect of actin microfilament disruption on TGF-β1 and EGF related changes in ^{3}H-thymidine incorporation.* Pretreatment of proximal tubule cells in culture with cytochalasin B prior to TGF-β1 addition, at a dose which retarded TGF-β1 induced transformation, ameliorated the decrease in EGF promoted ^{3}H-thymidine incorporation induced by TGF-β1. $^*P < 0.01$ compared to EGF alone; $^{**}P < 0.02$ compared to EGF and TGF-β1, $N = 6$ for each condition.

TGF-β1 in these cells. Both xyloside and galactosamine were used as proteoglycan synthesis inhibitors [20–22]. Xyloside inhibits proteoglycan production by competing with xylosylated core proteins for GAG elongation. The biosynthetic labeling of proteoglycans with ^{35}S-sulfate demonstrated this effect with a decline in radiolabeling of newly synthesized proteoglycans both in the supernatant and cell layer and an accumulation in the supernatant of low molecular weight GAGs. Galactosamine inhibits proteoglycan synthesis via a different mechanism by acting as a sink for UDP which is a necessary factor for saccharide incorporation into proteoglycans. Similar to xyloside, galactosamine was shown to inhibit TGF-β1 stimulated production of proteoglycans. This inhibitory effect was accompanied with attenuation of the TGF-β1-promoted phenotypic transformation of proximal tubule cells, as assessed by quantitative morphometry. Further support for a role for proteoglycans in this transformation response is the observation that simultaneous administration of uridine with galactosamine antagonized the effect of galactosamine on TGF-β1 transforming effects on the cells. This delay and inhibitory effects of these compounds suggest that proteoglycan synthesis is necessary for this transformation process. The fact that the inhibition is not complete suggests that other cellular elements are likely critical in this process.

Since this phenotypic change promoted by TGF-β1 on renal proximal tubule cells was characterized by both cellular aggregation and cellular migration, this change was also likely dependent upon cytoskeleton rearrangements. In this regard, assessment of TGF-β1 influences on actin microfilament structure in these cells with the utilization of rhodamine-conjugated phalloidin staining and immunofluorescent microscopy demonstrated that TGF-β1 provoked a dramatic rearrangement of actin microfilaments. Similar to other epithelial cells [24, 25], renal tubule cells in monolayer possess peripheral bands of actin near cell membranes and randomly oriented cytoplasmic fibers. Within a few hours following TGF-β1 treatment, actin fibers coalesced into stress fibers with direct linkages from cell to cell. Later these fibers were clearly seen to terminate at clearly-defined adhesion plaques. These effects of TGF-β1 on actin have been previously observed in other cell systems [28, 29]. The importance of this actin microfilament rearrangement in the TGF-β1 promoted phenotypic alteration was demonstrated with the use of cytochalasin B or D. These actin microfilament disrupting agents [30, 31] clearly attenuated stress fiber formation promoted by TGF-β1 and inhibited the TGF-β1-induced morphologic transformation of these cells.

These results demonstrate the importance of both proteoglycan synthesis and cytoskeleton rearrangement in the induction of the adhesive migratory response of these mesenchymally-derived epithelial cells promoted by TGF-β1. This response may be important in the cell biology of tissue modeling during wound repair and embryogenesis [32, 33].

Besides inducing a dramatic phenotypic transformation in renal epithelial cells, TGF-β1 has also been shown to influence the cell proliferative response of these renal tubule cells under both basal and mitogenic stimulation [13, 14]. This response has displayed both an early phase of enhancing cell proliferation and a later phase of inhibiting cell proliferation in renal tubule cells. Furthermore, the anti-proliferative effect of TGF-β1 on these cells was directly correlative to the phenotypic transformation promoted by this regulatory peptide, suggesting that the cellular elements responsible for this transformation may also be important in this anti-proliferative effect. In this regard, further experiments were carried out to assess the role of proteoglycans and cytoskeleton on the effect of both EGF and TGF-β1 on DNA synthesis in these cells. These results clearly demonstrate that proteoglycan synthesis is required to allow for a maximal proliferative response of these epithelial cells to the potent mitogen, EGF. This observation provides further evidence for a role for extracellular matrix molecules to regulate cell growth rates to various growth factors. In fact, TGF-β1 has been previously shown to potentiate the mitogenic effect of EGF on renal tubule cells during short exposure times [14]. The present results, which demonstrate that TGF-β1 promotes proteoglycan production by tubule cells and that proteoglycan synthesis inhibition attenuates EGF effects on cell proliferation, suggest that TGF-β1-induced proteoglycan production may be important in its early effects to stimulate EGF induced mitogenesis. Further experiments are required to determine whether the mechanism of this effect is due to changes in the affinity or number of cell surface receptors to EGF or due to alterations in the intracellular transduction process.

In contrast, actin microfilament disruption with cytochalasin B or D did not change the rate of DNA synthesis of these cells in response to EGF but did attenuate the anti-proliferative effect of TGF-β1 of these cells to EGF. Recent evidence has suggested that the mechanism of the anti-proliferative effect of TGF-β relates to influences on the proteins encoded by the c-*myc* and retinoblastoma genes [34, 35], although dissociation between myc-gene expression and growth inhibition by TGF-β1 in some cells has been reported [36, 37]. The current results provide a suggestion that an intact system for actin microfilament assembly is also required for the appropriate transduction of the TGF-β1 signal to promote a full anti-proliferative effect. Actin microfilament disruption clearly inhibited the TGF-β1

promoted phenotypic transformation of these cells and simultaneously attenuated the inhibitory effect of TGF-β1 on EGF-stimulated DNA synthesis. The role of the cytoskeleton in the transduction of peptide hormone signals is just beginning to be appreciated [38, 39].

In summary, TGF-β1 promotes the production of heparan sulfate proteoglycans and fibronectin and also promotes a well-defined progression of actin microfilament aggregation in renal proximal tubule cells in primary culture. Both the production of proteoglycans and the higher ordered structure of the cytoskeleton is instrumental in the adhesive, migratory response of these cells to TGF-β1 as well as the DNA synthesis rate responses to both EGF, as a cell growth promoter, and TGF-β1, as a growth inhibitor. These cellular processes are likely important in the effect of TGF-β1 to influence tissue modeling during tissue repair following injury and morphogenesis during embryonic development.

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